

## CLAIMS

WHAT IS CLAIMED IS:

1. A composition comprising an orthogonal leucyl-tRNA (leucyl-O-tRNA), wherein the leucyl O-tRNA comprises an anticodon loop comprising a CU(X)<sub>n</sub> XXXAA sequence, and comprises at least about a 25% suppression activity in presence of a cognate synthetase in response to a selector codon as compared to a control lacking the selector codon.
2. The composition of claim 1, wherein the leucyl-O-tRNA comprises a stem region comprising matched base pairs and a conserved discriminator base at position 73 and wherein the selector codon is amber codon.
3. The composition of claim 2, wherein the CU(X)<sub>n</sub> XXXAA sequence comprises CUCUAAA sequence and n=0.
4. The composition of claim 2, wherein the leucyl-O-tRNA comprises a C:G base pair at position 3:70.
5. The composition of claim 1, wherein the leucyl-O-tRNA comprises: a first pair selected from the group consisting of: U28:A42, G28:C42 and C28:G42; and, a second pair selected from the group consisting of: G:49:C65 or C49:G65; and, wherein the selector codon is a four-base codon.
6. The composition of claim 5, wherein the CU(X)<sub>n</sub> XXXAA sequence comprises a CUUCCUAA sequence and n=1.
7. The composition of claim 5, wherein the first pair is C28:G42 and the second pair is C49:G65.
8. The composition of claim 1, wherein the CU(X)<sub>n</sub> XXXAA sequence comprises a CUUCAAA sequence and n=0, and wherein the selector codon is an opal codon.
9. The composition of claim 1, wherein the leucyl-O-tRNA comprises or is encoded by a polynucleotide sequence as set forth in any one of SEQ ID NO.: 3, 6, 7 or 12, or a complementary polynucleotide sequence thereof.
10. The composition of claim 1, wherein the leucyl-O-tRNA and cognate synthetase, or a conservative variant thereof, are at least 50% as effective at suppressing a

selector codon as a leucyl O-tRNA of SEQ ID NO: 3, 6, 7 or 12, in combination with a cognate synthetase.

11. The composition of claim 1, further comprising an orthogonal leucyl aminoacyl-tRNA synthetase (leucyl O-RS), wherein the leucyl O-RS preferentially 5 aminoacylates the leucyl-O-tRNA with a selected amino acid.

12. The composition of claim 11, wherein the leucyl O-RS, or a portion thereof, is encoded by a polynucleotide sequence as set forth in any one of SEQ ID NO.: 13 or 14, or a complementary polynucleotide sequence thereof.

13. The composition of claim 11, wherein the leucyl O-RS comprises an amino 10 acid sequence as set forth in any one of SEQ ID NO.: 15 or 16, or a conservative variation thereof.

14. The composition of claim 1, wherein the leucyl-O-tRNA is derived from an archaeal tRNA.

15. The composition of claim 1, wherein the leucyl-O-tRNA is derived from 15 *Halobacterium sp NRC-1*.

16. The composition of claim 1, further comprising a translation system.

17. A cell comprising a translation system, wherein the translation system comprises:

an orthogonal leucyl-tRNA (leucyl-O-tRNA), wherein the leucyl-O-tRNA 20 comprises at least about a 25% suppression activity in presence of a cognate synthetase in response to a selector codon as compared to a control lacking the selector codon;

an orthogonal aminoacyl-leucyl-tRNA synthetase (leucyl-O-RS); and,

a first selected amino acid;

wherein the leucyl O-tRNA comprises an anticodon loop comprising a CU(X)<sub>n</sub>

25 XXXAA sequence and recognizes the first selector codon, and the leucyl O-RS preferentially aminoacylates the leucyl O-tRNA with the first selected amino acid.

18. The cell of claim 17, wherein the leucyl-O-tRNA comprises or is encoded by a polynucleotide sequence as set forth in any one of SEQ ID NO.: 3, 6, 7 or 12, or a complementary polynucleotide sequence thereof, and wherein the leucyl O-RS comprises an 30 amino acid sequence as set forth in any one of SEQ ID NO.: 15 or 16, or a conservative variation thereof.

19. The cell of claim 17, wherein the leucyl-O-tRNA and cognate synthetase, or a conservative variant thereof, are at least 50% as effective at suppressing a selector codon as a leucyl O-tRNA of SEQ ID NO: 3, 6, 7 or 12, in combination with a cognate synthetase.

20. The cell of claim 17, wherein the cell further comprises an additional different O-tRNA/O-RS pair and a second selected amino acid, wherein the O-tRNA recognizes a second selector codon and the O-RS preferentially aminoacylates the O-tRNA with the second selected amino acid.

21. The cell of claim 17, wherein the leucyl O-tRNA is derived from *Halobacterium sp NRC-1* and the leucyl O-RS is derived from *Methanobacterium thermoautotrophicum*.

22. The cell of claim 17, wherein the cell is a eukaryotic cell.

23. The cell of claim 17, wherein the cell is a non-eukaryotic cell.

24. The cell of claim 23, wherein the non-eukaryotic cell is an *E. coli* cell.

25. The cell of claim 17, further comprising a nucleic acid that comprises a polynucleotide that encodes a polypeptide of interest, wherein the polynucleotide comprises or encodes a selector codon that is recognized by the leucyl O-tRNA.

26. An *E. coli* cell comprising:  
an orthogonal leucyl-tRNA (leucyl-O-tRNA), wherein the leucyl-O-tRNA comprises at least about a 25% suppression activity in presence of a cognate synthetase in response to a selector codon as compared to a control lacking the selector codon;  
an orthogonal leucyl aminoacyl- tRNA synthetase (leucyl-O-RS), wherein the leucyl O-RS preferentially aminoacylates the leucyl O-tRNA with a selected amino acid;  
the selected amino acid; and,  
a nucleic acid that comprises a polynucleotide that encodes a polypeptide of interest, wherein the polynucleotide comprises a selector codon that is recognized by the leucyl O-tRNA, and wherein the leucyl O-tRNA is derived from *Halobacterium sp NRC-1* and the leucyl O-RS is derived from *Methanobacterium thermoautotrophicum*.

27. A polynucleotide selected from the group consisting of:  
(a) a polynucleotide comprising a nucleotide sequence as set forth in any one of SEQ ID NO.: 1-2, 4-7, 12;  
(b) a polynucleotide that is complementary to or that encodes a polynucleotide sequence of (a);

- (c) a nucleic acid that hybridizes to a polynucleotide of (a), or (b), under highly stringent conditions over substantially the entire length of the nucleic acid;
- (d), a polynucleotide that is at least 90% identical to that of a naturally occurring leucyl tRNA or a consensus leucyl-tRNA comprising SEQ ID NO: 12 and comprises an 5 anticodon loop comprising a CU(X)<sub>n</sub> XXXAA sequence, a stem region lacking noncanonical base pairs and a conserved discriminator base at position 73;
- (e) a polynucleotide that is at least 90% identical to that of a naturally occurring leucyl tRNA and comprises an anticodon loop comprising a CUUCCUAA sequence, a first pair selected from the group consisting of T28:A42, G28:C42 and C28:G42, and a second 10 pair selected from G:49:C65 or C49:G65.
- (f) a polynucleotide that is at least 98% identical to a polynucleotide of (a), (b), (c), (d), or (e); and,
- (g) a polynucleotide comprising a conservative variation of (a), (b), (c), (d), (e), or (f).
- 15 28. A vector comprising or encoding a polynucleotide of claim 27.
29. The vector of claim 28, wherein the vector comprises a plasmid, a cosmid, a phage, or a virus.
30. The vector of claim 28, wherein the vector is an expression vector.
31. A cell comprising the vector of claim 28.
- 20 32. A method of producing an orthogonal tRNA (O-tRNA), the method comprising:
- mutating an anticodon loop on members of a pool of tRNAs to allow recognition of a selector codon, thereby providing a plurality of potential O-tRNAs;
- analyzing secondary structure of at least one member of the plurality of potential O- 25 tRNAs to identify non-canonical base pairs in the secondary structure, and, optionally, mutating the non-canonical base pairs; and,
- subjecting to negative selection a first population of cells of a first species, wherein the cells individually comprise at least one member of the plurality of potential O-tRNAs, thereby eliminating cells that comprise a member of the plurality of potential O-tRNAs that 30 is aminoacylated by an aminoacyl-tRNA synthetase (RS) that is endogenous to the cell, and providing a pool of tRNAs that are orthogonal to the cell of the first species.
33. The method of claim 32, wherein the pool of tRNAs is derived from a species other than the first species.

34. The method of claim 32, wherein the pool of tRNAs is derived from at least a second species.

35. The method of claim 32, wherein the pool of tRNAs comprises one or more leucyl tRNAs.

5 36. The method of claim 32, wherein the pool of tRNAs is obtained by:

determining a consensus sequence;

generating a library of mutant tRNAs using the consensus sequence, thereby providing the pool of tRNAs.

10 37. The method of claim 32, further comprising subjecting to positive selection a second population of cells of the first species, wherein the cells comprise a member of the pool of tRNAs that are orthogonal to the cell of the first species, a cognate aminoacyl-tRNA synthetase, and a positive selection marker, to select or screen for cells that comprise a member of the pool of tRNAs that is aminoacylated by the cognate aminoacyl-tRNA synthetase and that shows a desired response in the presence of the positive selection marker, thereby providing an O-tRNA.

15 38. The method of claim 32, wherein the non-canonical base pairs are mutated to canonical base pairs.

20 39. The method of claim 32, wherein the non-canonical base pairs are located in stem region of the secondary structure.

40. The method of claim 32, further comprises adding a CCA sequence to a 3' terminus of one or more of the plurality of potential O-tRNAs.

41. The method of claim 32, wherein the selector codon comprises an amber codon, an opal codon or a four base codon.

25 42. The method of claim 32, further comprises measuring suppression activity.

43. The method of claim 32, wherein the subjecting step comprises expressing a polynucleotide that encodes a negative selection marker in the cell.

44. The method of claim 43, wherein the polynucleotide that encodes the negative selection marker comprises at least one selector codon.

30 45. The method of claim 44, wherein the polynucleotide encodes  $\beta$ -lactamase or  $\beta$ -galactosidase.

46. The method of claim 32, wherein the subjecting step comprises growing the population of cells in the presence of an selective agent.

47. The method of claim 46, wherein the selective agent comprises ampicillin.

5 48. The method of claim 43, wherein the negative selection marker fluoresces or catalyzes a luminescent reaction in the presence of a suitable reactant.

49. The method of claim 48, wherein a product of the negative selection marker is detected by fluorescence-activated cell sorting (FACS), or by luminescence.

50. The method of claim 43, wherein the negative selection marker comprises an affinity based screening marker.

10 51. An O-tRNA produced by the method of claim 32.

52. A method for identifying an orthogonal aminoacyl-tRNA synthetase for use with an O-tRNA, the method comprising:

subjecting to positive selection a population of cells of a first species, wherein the cells comprise: 1) a member of a plurality of aminoacyl-tRNA synthetases (RSs), wherein the plurality of RSs comprise mutant RSs, RSs derived from a species other than the first species or both mutant RSs and RSs derived from a species other than the first species; 2) an orthogonal tRNA (O-tRNA) from a second species; and 3) a polynucleotide that encodes a positive selection marker and comprises at least one selector codon; wherein cells that show an enhanced suppression efficiency as compared to cells lacking, or with a reduced amount 15 of the member of the plurality of RSs, comprise an active RS that aminoacylates the O-tRNA;

20 comparing a level of aminoacylation by the active RS of a first set of tRNAs from the first species to the level of aminoacylation by the active RS of a second set of tRNAs from the second species; wherein the level of aminoacylation is determined by a detectable 25 substance; and,

selecting the active RS that more efficiently aminoacylates the second set of tRNAs compared to the first set of tRNAs, thereby providing the orthogonal aminoacyl-tRNA synthetase for use with the O-tRNA.

30 53. The method of claim 52, wherein the aminoacylation is in vitro.

54. The method of claim 52, wherein the aminoacylation is in vivo.

55. The method of claim 52, wherein the detectable substance is a labeled amino acid.

56. The method of claim 52, wherein the O-tRNA comprises a leucyl O-tRNA.

57. The method of claim 56, wherein the leucyl O-tRNA comprises at least about a 25% suppression activity in presence of a cognate synthetase in response to a selector codon as compared to a control lacking the cognate synthetase.

5 - 58. The method of claim 52, wherein the plurality of RSs are derived from at least one leucyl RS.

59. An orthogonal aminoacyl-tRNA synthetase identified by the method of claim 52.

10 60. A method of producing a protein in a cell with a selected amino acid at a specified position, the method comprising:

growing, in an appropriate medium, the cell, where the cell comprises a nucleic acid that comprises at least one selector codon and encodes a protein; and,

providing the selected amino acid;

wherein the cell further comprises:

15 an orthogonal leucyl-tRNA (leucyl-O-tRNA) that functions in the cell and recognizes the selector codon; wherein the leucyl-O-tRNA comprises at least about a 25% suppression activity in presence of a cognate synthetase in response to a selector codon as compared to a control lacking the cognate synthetase; and,

20 an orthogonal aminoacyl-tRNA synthetase (O-RS) that preferentially aminoacylates the leucyl-O-tRNA with the selected amino acid.

61. A protein produced by the method of claim 60.